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(54) Title: TAXONOMIC IDENTIFICATION OF MICROORGANISMS, PROTEINS AND PEPTIDES INVOLVED IN VERTEBRATE DISEASE STATES

fluorescence pattern
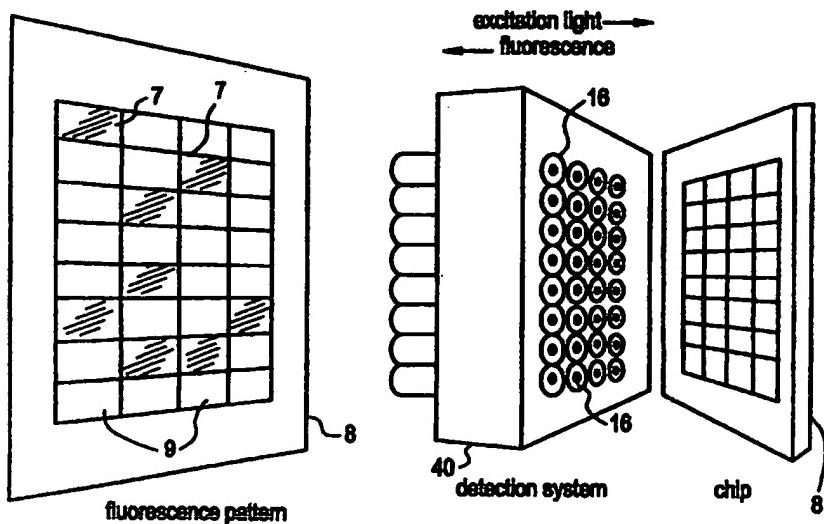
(57) Abstract

Method and apparatus for use in the identification of microorganisms, proteins and peptides in which a microorganism containing sample is contacted with a sensor chip having on a surface thereof a patterned array of a plurality of sections, each section having bonded thereto a ligand capable of binding a microorganism, protein or peptide. A number of different ligands are bonded to the various sections of the sensor chip, and thus serve to capture the microorganism, protein or peptide. Electromagnetic radiation is directed to the surface to ascertain which of the sections contains a microorganism, protein or peptide captured thereon, and then the microorganism, protein or peptide is identified as a function of one or more different ligands having a microorganism, protein or peptide bonded thereto.

TAXONOMIC IDENTIFICATION OF MICROORGANISMS, PROTEINS AND PEPTIDES INVOLVED IN VERTEBRATE DISEASE STATES

Background Of The Invention

The present invention relates to a method and apparatus for the taxonomic identification of microorganisms, and more particularly to the taxonomic identification of pathogenic microorganisms.

Pathogenic microorganisms, particularly pathogenic bacteria which either occur naturally or which have acquired virulence factors, are responsible for many of the diseases which plague mankind. Many of these bacteria have been proposed as biowarfare agents in the past. In addition, there is also the risk and likelihood that nonpathogenic microbes could also be used after genetic manipulation (e.g., Escherichia coli harboring the cholera toxin).

Typical pathogenic bacteria include those responsible for botulism, bubonic plague, cholera, diphtheria, dysentery, leprosy, meningitis, scarlet fever, syphilis and tuberculosis, to mention a few. During the last several decades, the public perception has been one of near indifference in industrialized

nations, principally because of successes that have been

achieved in combating these diseases using antibiotic therapy.

However, bacteria are becoming alarmingly resistant to

antibiotics. In addition, there have been recent revelations of

new roles that bacteria perform in human diseases such as

Heliobacteria phylori as a causitive agent of peptic ulcers,

Burkholderia cepaccia as a new pulmonary pathogen and Chlamydia

pneumoniae as a possible trigger of coronary heart disease.

Apart from those pathogens, various socioeconomic changes are

similarly contributing to the worldwide rise in food-borne

infections by bacteria such as Escherichia coli, Salmonella

spp., Vibrio spp., and Campylobacter jejuni.

Potential infections are also important considerations

in battlefield medicine. A number of bacterial pathogens,

including Bacilis anthracis and Yersininia pestis and their

exotoxins, have been used as weapons in the past. And, as

noted, there is always the increasing risk that nonpathogenic

microbes can be engineered to be pathogenic and employed as

biowarfare agents.

Pathogenic microorganisms are also of concern to the

livestock and poultry industries as well as wildlife management.

For example, Brucella abortis causes the spontaneous abortion of

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calves in cattle. Water supplies contaminated with exotoxin-

producing microorganisms have been implicated in the deaths of

bird, fish and mammal populations. More recently, mad cow

disease has been traced to the oral transmission of a

proteinaceous particle not retained by filters. Thus, there is

clearly a need for the rapid and inexpensive techniques to

conduct field assays for toxic proteins and pathogenic

microorganisms that plague animals as well as humans.

As a general proposition, bacterial contamination can

be detected by ordinary light microscopy. This technique,

however, is only of limited taxonomic value. The investigation

and quantitation of areas greater than microns in size are

difficult and time consuming. Many commercially available

systems rely on the growth of cultures of bacteria to obtain

sufficiently large samples (outgrowth) for the subsequent

application of differential metabolic tests for species (genus)

identification. However, techniques requiring bacterial

outgrowth may fail to detect viable but nonculturable cells. To

the contrary, the growth media employed may favor the growth of

bacteria with specific phenotypes.

More sensitive and more rapid typing schemes are

described in "Strategies to Accelerate the Applicability of Gene

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Amplification Protocols for Pathogen Detection in Meat and Meat

Products" by S. Pillai and S.C. Ricke and "Molecular Approaches

for Environmental Monitoring of Microorganisms" by R.M. Atlas, G.

Sayle, R.S. Burlage, and A.K. Bej. Those techniques employ the

polymeric chain reaction (PCR) for amplification of bacterial

DNA or RNA, followed by nucleic acid sequencing to detect the

presence of a particular bacterial species. Such general

amplification and sequencing techniques require technical

expertise and are not easily adaptable outside of specialized

laboratory conditions. Moreover, such PCR methodology cannot

indicate whether the target bacterium was viable; this technique

provides a positive analysis whenever an intact target nucleic

acid sequence is detected.

Another approach utilizes immunochemical capture as

described in "The Use of Immonological Methods to Detect and

Identify Bacteria in the Environment" by M. Schlotter, B. Assmus

and A. Hartmann Biotech. Adv. 13, 75, followed by optical

detection of the captured cells. The most popular immunoassay

method, enzyme-length immunosorbent assay (ELISA), has· a

detection limit of several hundred cells. That is well below

the I.D.$_{50}$ of extremely infectious bacteria such as Shigella

flexneri. Piezoelectric detection techniques, such as those

described by "A Piezoelectric Biosensor for Listeria

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Monocytogenes" by M.B. Jacobs, R.M. Carter, G.J. Lubrano and G.G.
Guilbault, are even less sensitive having a detection limitation
of about 5 x 10^5 cells. A recent report entitled "Biosensor
Based on Force Microscope Technology" by D.R. Baselt, G.U. Lee
and R.J. Colton describes the use of an atomic force microscope
(AFM) to detect immunocaptured cells. Like other immunoassay
techniques, viable cells cannot be discriminated from dead cells
because cell capture and detection is predicated on the presence
of an intact bacterial antigen. Immunoassays are also presently
used in the trace analysis of peptides and proteins.

Moreover, the prior art has made extensive use of
immobilized antibodies in peptide/protein/microorganism capture.
Those techniques likewise involve significant problems because
the antibodies employed are very sensitive to variations in pH,
ionic strength and temperature. Antibodies are likewise
susceptible to degradation by a host of proteolytic enzymes in
"dirty" samples. In addition, the density of antibody molecules
supported on surfaces (e.g., microwell plates or magnetic beads)
is not as high as is frequently necessary.

Medical and military considerations call for better
toxin and pathogen detection technologies. Real-time assessment
of battlefield contamination by a remote sensing unit is

necessary to permit and facilitate rapid diagnosis to permit

appropriate counter-measures. A microbe/toxic protein sensor

useful in such situation requires the ability to globally

discriminate between pathogens and nonpathogens as well as

discriminate between viable cells (including spores) and dead

cells. In addition, such techniques require high sensitivity

when ten or fewer cells are present and analysis that can be

completed in the field in less than 15 minutes. In cases such

as this, even in pyrolysis, followed by mass spectral analysis

of volatile cell components (e.g., fatty acids) would be

extremely difficult and hence impractical for routine assays.

Such techniques should be able to recognize pathogens and

provide some assessment of strain virulence or toxigenicity.

In copending application Serial No. 559,043, filed

June 3, 1996, the disclosure of which is incorporated herein by

reference, there is described a method and apparatus for sensing

the presence of microbes on a non-living surface which is

particularly well-suited to detect the presence of microbes in

meat, poultry and like food products. In accordance with the

system described in the foregoing copending application,

microbes which may be present on non-living surfaces such as

meat and poultry are subjected to electromagnetic energy having

wavelengths greater than about 350 nm. The electromagnetic

radiation excites the microbial cells present on the surface to emit electromagnetic energy (i.e., fluoresce) having a wavelength greater than that of the excitation wavelength. Any microbial cells present on the surface containing reduced pyridine nucleotides such as nicotinamide adenine dinucleotide (NADH) will emit a characteristic fluorescence signal. The presence of living microbial cells is determined by sensing both the fluorescence from the cell respiration and electromagnetic radiation reflected or scattered by the surface.

While the system disclosed in the foregoing application represents a significant advance in the art in detecting the presence of living microbial cells, it cannot be used as such to taxonomically evaluate the microbes present on the surface, or identify proteinaceous toxins or peptide hormones of pathophysiological importance to vertebrate animals, including humans, livestock, poultry and wildlife.

It is accordingly an object of the present invention to provide a method and apparatus for taxonomically evaluating microbes, proteins or peptides which overcome the foregoing disadvantages.

It is a more specific object of the invention to

provide a method and apparatus for taxonomically evaluating microbes, proteins and peptides which have the capability of discriminating between pathogens and nonpathogens and viable cells from dead cells and can be likewise used to identify proteins or peptides.

It is yet another object of the invention to provide a method and apparatus for taxonomically evaluating microbial cells characterized by high sensitivity (ten to one hundred cells) and which can taxonomically identify microbes under field conditions and can likewise be used to identify trace amounts of proteins or peptides.

Summary Of The Invention

The concepts of the present invention reside in a method and apparatus for the taxonomic identification of microorganisms in which microbes are captured through the binding of microbial receptors to specific ligands tethered to a surface, and the electromagnetic radiation is used to determine the presence of metabolites or other characteristic biomolecules for the detection of the presence of the captured microorganisms in accordance with the practice of the invention, a microorganism-containing sample is contacted with a sensor chip,

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the sensor chip having a patterned area on its surface

containing a plurality of sections, with each section having

bonded thereto a ligand capable of bonding to a specific

microbial receptor. The receptor may be, for example, a protein

residing in the outer membrane of the microbial cell, pilus or

flagellum which is exposed to the aqueous environment

surrounding a cell. In accordance with the concepts of the

present invention, the same receptor likewise forms the basis

for the detection of peptides and proteins of pathological

interest.

Electromagnetic radiation is directed onto the surface

of the sensor chip to excite sections of the sensor chip to

determine which of the sections of the sensor chip contain a

microorganism binding to the ligand on that particular section.

By determining which sections have a microorganism bound to a

ligand, it is possible to taxonomically identify the

microorganism contained in the sample as a function of the

combination of different ligands which have a microbe bound

thereto.

Thus, the method of the present invention does not

depend on classical antigen-antibody recognition. On the

contrary, the concepts of the present invention make use of

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relatively inexpensive reagents in the capture of

microorganisms, peptides or proteins contained in the sample.

        The sensor chip employed in the practice of the

present invention are preferably formed from a suitable support

material such as glass or non-reactive plastic substrates such

as polystyrene and polymethylmethacrylate. The sensor chip is

formed of a patterned array defining a plurality of sections on

the surface of the sensor chip, and each section has bonded

thereto a different ligand capable of molecularly recognizing a

specific peptide, protein or microbial receptor and hence the

microbe itself. The ligand for pathogen/peptide/protein capture

bonded to the surface of the sensor chip can and should be

varied. In general, such ligands may be characterized as heme,

siderophores, oligosaccharides and anti-adhesion peptides

capable of capturing a wide variety of microorganisms, toxic

proteins and peptides. Those ligands can thus be immobilized or

bonded to the surface of the sensor chip by means of organic

coupling agents having the capability of reacting with the

surface of the sensor chip itself and also having the capability

of reacting with the ligands whereby the coupling agent

establishes a chemical bond or "tether" between the surface of

the sensor chip and the ligand capable of reaction with a

variety of different microorganisms, proteins and/or peptides.

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Particularly useful in the bonding of the ligands to glass

sensor chip substrates are the organosilanes having 1-3 readily

hydrolyzable groups attached directly to the silicon atom and a

functional organic group also attached to the silicon atom, the

functional group being capable of reaction with the ligand.

In the preferred practice of the invention, the

patterned array of the sensor chip is preferably positioned on

the surface thereof in a pattern such as rows of sections. Each

one of the sections, in the preferred practice of the invention,

is exposed to electromagnetic radiation to excite captured

protein, peptide or biomolecule present in the captured microbes

having characteristic emission fluorescence. That fluorescence

can then be detected by a suitable apparatus for detecting

electromagnetic radiation and converting that radiation into an

electrical signal as an indication of whether or not a

particular section has a microorganism, protein or peptide

bonded to the ligand for that section. In the preferred

practice of the invention, the probe, which may be a probe like

that described in the foregoing copending application, can be

sequentially positioned to direct electromagnetic radiation to

each of the sections in turn. Alternatively, each section of

the sensor chip can be scanned simultaneously. The output of

the fluorescence detector can then be converted to an electrical

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signal indicative of those sections having a microorganism,

protein or peptide bonded thereto. The microorganism, protein

or peptide present can thus be identified by examining which of

the sections have captured the species of interest and which

have not.

Thus, the present invention can be rapidly used to

identify microorganisms without the need for growing a culture

of the microorganism and then microscopically examining the

culture thus produced. Likewise, low levels of toxic proteins

or peptide hormones can similarly be identified. It is also

unnecessary to employ enzymes or antibodies in the capture of

microbial metabolites as is often used in the prior art.

Brief Description Of The Drawings

Fig. 1 is a schematic illustration showing the sensor

chip employed in the practice of the invention, illustrating the

different sections contained on the face thereof having

different ligands attached to each section.

Fig. 2 is a schematic illustration of a multiple

element detection system which can e used in the practice of the

present invention.

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Fig. 3 is a graph illustrating the fluorescence

excitation (EX) and emission (EM) spectrum of a sample of meat

with and without E coli contamination and meat with fat having

no E coli contamination. Contamination was approximately 10^3

cells/cm^2.

Fig. 4 is a graph illustrating fluorescence excitation

(EX) and emission (EM) spectrum of tryptophan (Trp), tyrosine

(Tyr) and calcium dipicolinate (DP) in Bacillus cereus spores.

Fig. 5 illustrates schematically a system embodying

the present invention for scanning a sensor chip in the practice

of the invention.

Detailed Description Of The Invention

In the practice of the present invention, a sample

containing an unknown analyte microorganism, protein or peptide

is first contacted with the sensor chip. The sensor chip is

illustrated in Figure 1 of the drawings and is formed of a

substrate 8 such as a glass slide having a series of sections

formed thereon, each of which having a series of sections 1

through 70 on the surface thereof. Each section has a different

ligand bonded thereto so as to be capable of binding to specific

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analytes. The ligands are capable of binding to the analyte for

capture and the presence of the captured analyte is detected

using the fluorescence detection system disclosed and claimed in

copending application Serial No. 659,043, filed June 3, 1996.

Thus, the ligand of each of the sections of the sensor chip 8

has the capability of capturing a specific microorganism,

peptide or protein.

In the preferred practice of the invention, the

ligands used in the present invention are taken from the group

of heme compounds, siderophores, oligosaccharides and peptides.

As is well known to those skilled in the art, animal

pathogens generally possess heme uptake capability, and thus

heme compounds can be used to capture a number of pathogenic

species. In addition to heme compounds, other ligands in the

form of high-affinity iron chelators, generally referred to as

siderophores, can also be used to capture many strains of

pathogenic bacteria. Included among such siderophores are

alcaligin, mycobactins, pyochelin, staphyloferrin, vibriobactin

and yersiniabactin.

In addition to heme compounds and siderophores,

eukaryotic surface epitopes (peptides or carbohydrates) which

are recognized by microbial cell receptors, can likewise be used

as ligands in the practice of the present invention. These

ligands include commercially available oligosaccharides as well

as those available by chemical synthesis. Other

oligosaccharides and their affinity to pathogens from various

microorganisms are described by Karlsson "Microbial Recognition

of Target Cell Glycoconjugates", Structural Biology, 1995, 5:

622,635, the disclosure of which is incorporated herein by

reference.

        The characteristics of a number of bacterial species

along with the diseases caused by such bacteria and their

binding characteristics with siderophores, oligosaccharides and

hemin are set forth in Table I. These characteristics can be

used in the capture and identification of such species.

        Peptide ligands can be produced by affinity panning of

libraries of oligopeptides displayed on bacteriophages or on

Escherichia coli flagella. Such ligands are useful in the

capture of soluble proteins and peptide hormones as well as

microorganisms.

Table 1

Bacterial Species	Disease Caused	Siderophore Binding?	Oligosaccharide Binding?	Hemin Binding?	Exotoxin Produced?
Bacillus anthracis	anthrax	unknown	unknown	unknown	anthrax toxin
Bordetella pertussis	whooping cough	alcaligin, others	N-Acetyl-glucosamine	Yes	pertussis toxin
Clostridium botulinum	botulism	unknown	unknown	Yes	botulinum toxin
Clostridium perfringens	gas gangrene	unknown	unknown	unknown	α-toxin
Clostridium tetani	tetanus	unknown	unknown	unknown	tetanus toxin
Corynebacterium diphtheriae	diphtheria	aerobactin	unknown	unknown	diphtheria toxin
Escherichia coli 0157:H7	numerous infections	many	globobiose, others	Yes	Shiga-like toxin
Haemophilus Influenzae	meningitis	enterobactin	GalNAcβ(1-4)Gal, others	Yes	unknown
Helicobacter pylori	gastric ulcers	unknown	a mucosal oligosaccharide	unknown	vacuolating cytotoxin A
Klebsiella pneumoniae	numerous infections	many	GalNAcβ(1-4)Gal, others	Yes	unknown

Table 1 (cont.)

Bacterial Species	Disease Caused	Siderophore Binding?	Oligosaccharide Binding?	Hemin Binding?	Exotoxin Produced?
Mycobacterium tuberculosis	tuberculosis	mycobactin T	unknown	unknown	unknown
Neisseria meningitidis	meningitis	many	unknown	Yes	unknown
Pseudomonas aeruginosa	numerous infections	pyochelin, others	asialo G_{M1}, others	Yes	exotoxin A, others
Salmonella typhi	typhoid fever	many	unknown	Yes	Yes
Serratia marescens	numerous infections	aerobactin, ferrioxamine B	Yes	Yes	Yes
Shigella dysenteriae	dysentery	enterobactin, aerobactin	Yes	Yes	Shiga toxin
Staphylococcus aureus	numerous infections	staphyloferrin, others	$GalNAc\beta(1-4)Gal$	Yes	several superantigens
Streptococcus pneumoniae	pneumonia, meningitis	unknown	GlcNAcetyl-(1-3)Gal, others	Yes	Yes
Vibrio cholerae	cholera	vibriobactin, others	Yes	Yes	cholera toxin
Yersinia pestis	bubonic plague	yersiniabactin	unknown	Yes	YopE

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Toxins that contain at least one tryptophan or several
tyrosines per molecule can be detected by tryptophan/tyrosine
fluorescence after capture using a tethered peptide (produced by
biopanning a library of peptides). A variety of microbes,
including algae, fungi, protozoans, and bacteria export
exotoxins that are amenable to detection using this technology.
Selected tissues of a variety of higher plants yield toxic
proteins. A variety of animals, including reptiles, amphibians,
marine invertebrates, scorpions, spiders, and insects, produce
toxic proteins and peptides as well. The following list
contains examples of toxic proteins and peptides that can be
captured and detected using the technology described herein.

Ridinus communis (castor bean)   ricins
Apis mellifera (honey bee)   mellitin
Latrodectus mactans (black widow spider) α-latrotoxin
Agelenopsis aptera (funnel web spider)   ω-agatoxin TK
Bunodosoma granulifera (sea anemone)   K+-channel-blocking
                                           toxin
Bungarus multicinctus (krait)   β-bungarotoxin
Naja naja atra (Formosan cobra)   cobratoxins

Bacteria:
Bacillus anthracis   anthrax toxins (all
                                           components
Clostridium botulinum   botulinum toxins
Vibrio Cholerae   cholera toxin
Clostridium perfringens   α-toxin
                                           (phospholipase C)
Corynebacterium diphtheriae   diphtheria toxin
Escherichia coli   heat-labile enterotoxin
Bordetella pertussis   pertussis toxin
Shigella dysenteriae   Shiga toxin
Staphylococcus aureus   toxic shock syndrome

	toxin-1
Clostridium tetani	tetanus toxin
Yersinia pestis	YopE
Helicobacter pylori	vacuolating cytotoxin A

Examples of human bioactive peptides (including

peptide hormones) that can be detected using tryptophan/tyrosine

fluorescence:

adrenocorticotropic hormone (ACTH)
bombesin
gastrins
gastrin-releasing peptide (GRP)
neuropeptide Y (NPY)
luteinizing hormone releasing hormone (LH-RH)
β-melanocyte stimulating hormone
parathyroid hormone (PTH)
somatostatin
endothelins

The various ligands are preferably tethered to a

substrate by means of organic coupling agents which are

themselves well known to those skilled in the art. When using a

glass substrate for the sensor chip, it is frequently preferred

to employ, in the practice of the present invention,

organosilane compounds having the following general structure:

$$
\begin{array}{c}
R_1 \\
| \\
R_2 - Si - R_4 \\
| \\
R_3
\end{array}
$$
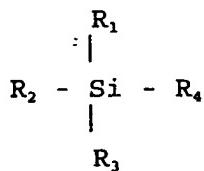
wherein R_1 through R_3 are each selected from the group consisting

of hydrogen, alkyl groups containing 1 to 6 carbon atoms, aryl

groups containing 6 to 12 carbon atoms and alkoxy groups

containing 1 to 4 carbon atoms, with at least one of R_1, R_2 and

R_3 being an alkoxy group. R_4 is an organic group containing at

least 3 carbon atoms and also containing a functional group

capable of reaction with the ligand. Without limiting the

invention, suitable organic groups are polyamines and polyethers

containing 3 to 30 carbon atoms. Also suitable for use in the

practice of the invention are coupling agents containing other

functional groups such as epoxy groups, amino groups and

unsaturated functional groups, OH groups, thiol groups and the

like, which are capable of reaction with the various ligands.

Without limiting the invention as to theory, it is believed that

the ligand reacts with the functional group, preferably a

terminal functional on the organosilane compound while the

readily hydrolyzable alkoxy group attached directly to the

silicon atom has the capability of reacting directly with the

surface of the glass substrate of the sensor chips. Thus, the

ligand is tethered to the surface of the glass through the

coupling agent.

Thus the ligand tethered to the glass surface may be

illustrated by the following:

        |

Glass-O-Si-$(CH_2)_3$-NH-$(CH_2)_3$-NH-$(CH_2)_5$-NH-$(CH_2)_{12}$-NH-$(CH_2)_5$-NJ-LIGAND

        |
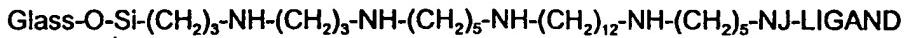
The chemical reactions used in tethering ligands to the surface

of the sensor chip are known to those skilled in the art and are

described in the literature. Such reactions may be found in

G.T. Hermanson, <u>Bioconjugate Techniques</u>, San Diego: Academic

Press, 1966; Hansson et al., "Carbohydrate-Specific Adhesion of

Bacteria to Thin Layer Chromatograms: A Rationalized Approach to

the Study of Host Cell Glycolipid Receptors", <u>Analytical</u>

<u>Biochemistry</u>, 146, 158-163 (1985); and, Nilsson et al., "A

Carbohydrate Biosensor Surface for the Detection of

Uropathogenic Bacteria", <u>Bio/Technology</u>, 12, 1376-1378, December

1994.

       Illustrative of such reactions are those used to

tether ferroxamine as a ligand to the surface of a glass sensor

chip. In the first stage, a glass surface containing free

hydroxyl groups is first reacted with a 2% solution of gamma-N-

(aminopropyl)-gamma-aminopropyltrimethoxysilane to attach the

silane to the glass surface:

$$Glass\text{-}OH + (CH_3O)_3\text{-}Si\text{-}(CH_2)_3\text{-}NH\text{-}(CH_3)_3\text{-}NH_2 \rightarrow$$

(I)

$$Glass\text{-}O\text{-}Si\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_3\text{-}NH$$
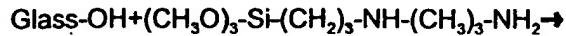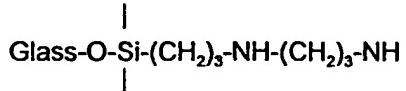
The product of that reaction can then be reacted with

glutaraldehyde at a pH of about 8 to form the corresponding

aldehyde:

$$Glass\text{-}O\text{-}Si\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_3\text{-}N=C\text{-}(CH_2)_3\overset{\overset{\displaystyle O}{\|}}{\text{-}C}\text{-}H$$

(II)
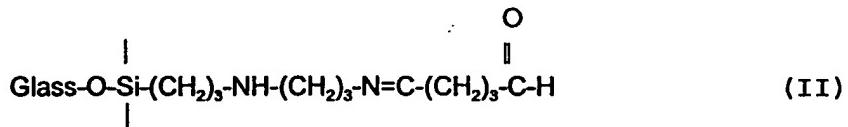
The aldehyde, in turn, can be reacted with a diamine:

$$H_2N\text{-}(CH_2)_{12}\text{-}NH_2$$

(III)

$$Glass\text{-}O\text{-}Si\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_3\text{-}\underset{\underset{\displaystyle H}{|}}{N} = C\text{-}(CH_2)_3\text{-}\underset{\underset{\displaystyle H}{|}}{C} = N\text{-}(CH_2)_{12}\text{-}NH_2$$

(IV)
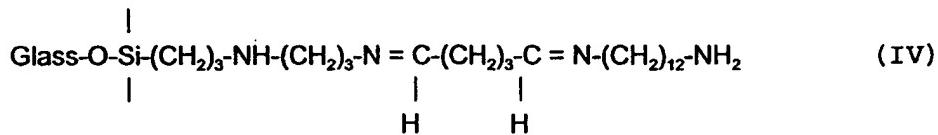
Next, the product of the preceding reaction is reacted

with glutaraldehyde to introduce a (terminal) aldehyde group:

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$$\text{Glass-O-Si-(CH}_2)_3\text{-NH-(CH}_2)_3\text{N = C-(CH}_2)_3\text{-C = N-(CH}_2)_{12}\text{-N = CH-(CH}_2)_3\text{-CHO} \qquad \text{(V)}$$
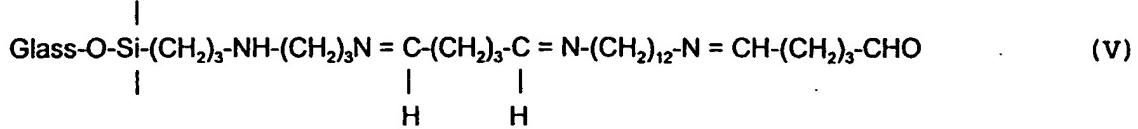
which can then be reduced using NaCNBH$_3$ to yield:

$$\text{Glass-O-Si-(CH}_2)_3\text{-NH-(CH}_2)_3\text{-NH-(CH}_2)_5\text{-NH-(CH}_2)_{12}\text{-NH-(CH}_2)_4\text{-CHO} \qquad \text{(VI)}$$
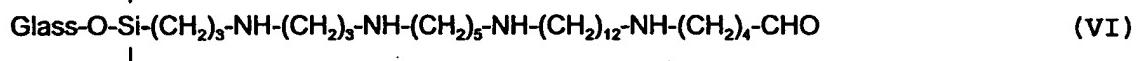
The foregoing silane coupling agent bonded to the surface can then be derivatized by reaction with deferrioxamine B (or DFA) at an alkaline pH to yield:

$$\text{Glass-O-Si-(CH}_2)_3\text{-NH-(CH}_2)_3\text{-NH-(CH}_2)_5\text{-NH-(CH}_2)_{12}\text{-NH-(CH}_2)_4\text{-CH = N-DFA} \qquad \text{(VII)}$$
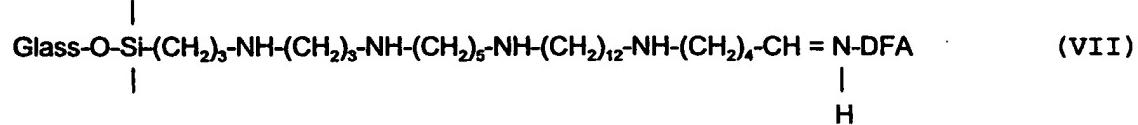
The DFA can then be complexed with Fe by reaction with a ferrous salt in aqueous medium to form the ligand.

As will be appreciated by those skilled in the art, many other techniques can likewise be used to tether an appropriate ligand to the surface of the sensor chip. For example, thiol-terminated peptides can be tethered to the surface of a glass sensor chip using similar reactions. For

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example, IV above can be reacted with N-(gamma-

alemidobutyryloxy) succinimide ester (GMBS) to form the

following derivative:

Glass -O-Si-(CH₂)₃-NH-(CH₂)₃-N=CH-(CH₂)₃-CH=N-(CH₂)₁₂-NH-CO-(CH₂)₃-N
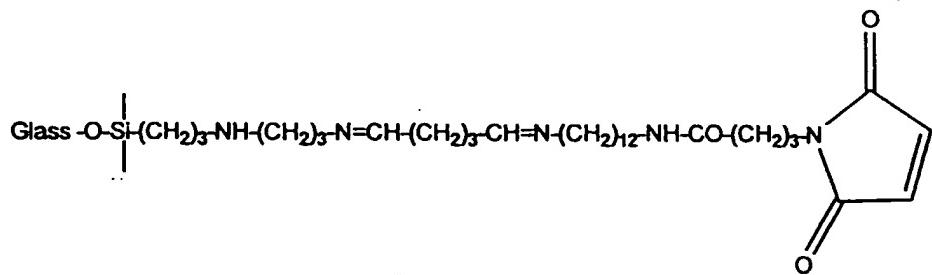
That derivative can, in turn, be sequentially reacted

with the thiol-terminated peptide to form the corresponding

peptide terminated compound. That, in turn, can, if desired, be

reduced using sodium cyanoborohydride to yield the following

peptide ligand tethered to the glass surface:

Glass -O-Si-(CH₂)₃-NH-(CH₂)₃-NH-(CH₂)₅-NH-(CH₂)₁₂-NH-CO-(CH₂)₃-N——S-Peptide
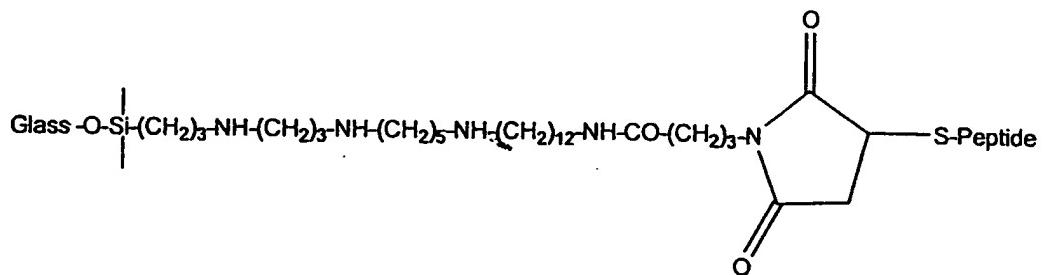
Similarly, the same overall reaction scheme can

likewise be used to tether hemin to the glass surface of the

sensor chip. Hemin, also known as ferriprotoprophyrin IX, can

be reacted with N-hydroxysuccinimide to form the hemin di(N-

hydroxysuccinimide) ester. That diester can then be reacted

with the product IV referred to above which has been previously

reduced with sodium cyanoborohydride to form the following

-25-
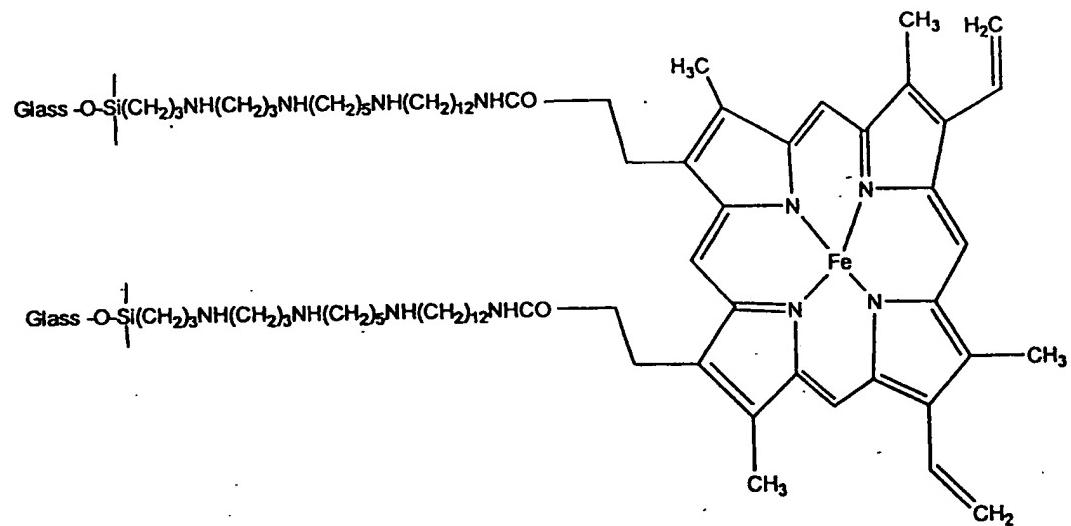
tethered ligand:

Thus, as described above, a different ligand is

tethered to each of the sections of the sensor chip. The sensor

chip is then contacted with a sample containing an unknown

organism, protein or peptide whereby specific ligands on the

surface of the chip bind to specific analytes, selectively

capturing them. The sensor chip is then subjected to

electromagnetic radiation using the equipment described in

Serial No. 659,043 so that each section of the sensor chip is

exposed to an appropriate wavelength of electromagnetic

radiation to excite fluorescence characteristic of the presence

of bound analytes. Appropriate fluorescence signals generated

by metabolites or other biomolecules specific to pathogenic

microorganisms on exposure to electromagnetic radiation are set

forth in Table 2. Note NAD[P]H is only present in viable or

respiring cells; calcium dipicolinate as a significant presence

in spores of the order of about 15% but is otherwise rare in

nature. The relative signals as measured in bacteria and spores

are shown in Figs. 3 and 4, respectively. Any proteinaceous

toxin or peptide hormone that contains tryptophan or tyrosine

will also generate fluorescence signals as noted in Table 2 for

these fluorophores.

        The sensor is illustrated in Fig. 5 of the drawing in

schematic form, and includes a source of light 10 having the

desired wavelength. As will be appreciated by those skilled in

the art, the source of electromagnetic radiation 10 can be any

of a variety of devices producing UV light having a wavelength

less than about 400 nm. For example, the source of

electromagnetic radiation 10 can be a laser or various types of

lamps emitting electromagnetic radiation generally within the UV

range.

        The wavelength of light is chosen by an excitation

filter 12, preferably in the form of a narrow band-width filter

passing electromagnetic radiation having wavelengths within the

desired range. For example, when seeking to excite NADH, the

filter 12 should be one which emits electromagnetic radiation

-27-

within the range of about 350 to 390nm and preferably with a

peak of about 366nm.

Positioned to receive the light emitted from the light

source 10 through filter 12 is a conduit for electromagnetic

radiation 14, preferably in the form of a fiber optic element or

bundle of elements, capable of conducting the electromagnetic

energy passing from the source 10 through the filter 12. The

fiber optic bundle terminates in a probe 16, which can be a

probe used to successively scan each of the sections of the

sensor chip, as in a rastor scan or in one element of a multi-

element probe system with one element for each section.

Also contained within the probe are a pair of

additional fiber optic elements or bundles 20 and 22 illustrated

in Fig. 2, positioned to transmit electromagnetic radiation from

the sensor chip 8 for detection. In the illustrated embodiment,

fiber optic element 20 conveys electromagnetic energy from the

sensor chip 8 to an emission filter 24; that filter is chosen to

pass electromagnetic radiation having the wavelength of the

fluorescence generated in the microbial cells, peptides or

proteins present on each of the sections of the sensor chip 8.

It is also desirable, in the practice of the

invention, to use another filter 26 associated with fiber optic

element 22 which passes only electromagnetic radiation having

substantially the same wavelength as the electromagnetic

radiation directed to the sensor chip through fiber optic

element 14. Thus, filter 26 should pass electromagnetic

radiation within the same range as that used for excitation.

The choice of excitation and emission filters is set forth in

Table 2. The filter combinations can be easily changed by

mounting the filters on a rotating wheel or sliding mechanism to

provide detection of all of the compounds in Table 2.

The apparatus shown in Fig. 5 also includes a pair of

detector elements 28 and 30 which detect electromagnetic

radiation passing through filters 24 and 26, respectively. As

will be appreciated by those skilled in the art, the detectors

28 and 30 are elements sensitive to electromagnetic radiation,

converting that radiation into an electrical signal which is

proportional to the intensity of the radiation presented to the

detectors through filters 24 and 26, respectively. Conventional

devices including photodiodes, photomultiplier, tubes, video

cameras, charge-coupled devices as well as other detectors may

be used.

Because the electromagnetic radiation passed through

the associated filter 24 is essentially limited to the

wavelength of the electromagnetic energy associated with the

fluorescence of a specific metabolite or other biomolecules, the

amount of fluorescence due to that metabolite passing through

the filter 24 from the sensor chip 8 and detected by the

fluorescence detector 28 is related to the amount of that

metabolite present and thus to the number of microorganism,

protein molecules or peptide molecules present. The

electromagnetic radiation passed through fiber optic element 22

is filtered to pass only electromagnetic radiation having a

wavelength substantially the same as that of the electromagnetic

energy directed into the sensor chip for that particular

section; detector 30 measures only the reflected radiation from

the sensor chip. Both detectors 28 and 30 thus convert the

electromagnetic energy to a corresponding electrical signal, and

the signal indicative of the presence of analytes of interest is

determined by dividing the amount of electromagnetic radiation

passing through filter 24 to detector 28 (representing the

fluorescence of any captured species present on that section of

the sensor chip) by the electromagnetic radiation passing

through filter 26 to the detector 30 (representing the reflected

electromagnetic radiation). This signal is then normalized by

subtraction of this ratio for the blank section of the sensor

chip. Thus, the signal may be represented by the difference

-30-

between the fluorescence divided by the reflectance for a

section containing a captured analyte of interest minus the

fluorescence divided by the reflectance for a blank section.

An alternate system for scanning the sensor chip 8 is

illustrated in Fig. 2 of the drawing showing a sensor chip 8

which has been contacted with an analyte. Various sections 9

contain no captured analyte thereon while other sections 7 do

have a captured analyte. It is possible, and sometimes

desirable, to determine the presence of a bound analyte in each

of the sections of the sensor chip 8 simultaneously.

That can be done using a system described in Fig. 2

having a matrix support member 40 which includes a plurality of

probes 16 mounted therein, with the probes 16 mounted in the

matrix 40 being patterned to correspond to the pattern of

sections in the chip 8.

In the preferred embodiment, the matrix 40 includes

one probe 16 for each of the sections on the sensor chip 8,

although, as will be understood by those skilled in the art,

either fewer or greater probes may be used under some

circumstances. The matrix is then positioned proximate to a

chip 8 which has been exposed to an unknown sample and each of

-31-

the sections is exposed to electromagnetic radiation

simultaneously. Accordingly, each of the sections is

simultaneously examined by electromagnetic radiation to detect

the presence of captured analytes on each section of the chip 8.

The system of Fig. 2 otherwise operates in the same manner as

that described in Fig. 5.

By comparing fluorescence signals to reflected

signals, the system as described in detail in the foregoing,

copending application, normalizes the signals. That allows the

system to compensate for variations in the distance of the probe

from the surface of the chip 8 and variations between different

surfaces. As will be appreciated by those skilled in the art,

it is possible, and sometimes desirable, to either use multiple

sources of electromagnetic radiation or to employ multiple

filters 12. In that way, the electromagnetic radiation directed

to a single section of the sensor chip 8 may be changed going

from one section of that chip to another section of the chip.

That technique thus permits different wavelengths of

electromagnetic radiation to be directed to different sections

of the sensor chip, depending on the ligand tethered to that

particular section of the sensor chip.

As will be appreciated by those skilled in the art, a

variety of bacterial cell components or metabolites as well as

proteins and peptides exhibit intrinsic fluorescence when

illuminated by UV light. For example, NADH has been extensively

used in the study of various organisms and can be employed to

determine whether a particular microbial sample contains viable

or respiring cells (Fig. 3). Similarly, tryptophan likewise

exhibits fluorescence indicative of spores, nonviable cells,

most protein toxins and many peptide hormones, while calcium

dipicolinate exhibits fluorescence indicative of the presence of

spores (Fig. 4). Those fluorophores, their excitation and

emission frequencies in vivo as well as the indications they

provide are set forth in Table 2:

Fluorophore	Excitation(nm) Range Peak	Emission(nm) Range Peak	Source
NAD[P]H	350-390 366	415-465 440	Viable[Respiring] Cells
tryptophan (tyrosine)	265-295 280 (274)	315-385 350 (295-315) (305)	Spores Non-Viable Cells Most Exotoxins Many Peptides
Ca++dipicolinate	280-310 295	380-460 420	Spores.

In the practice of the present invention, a sample

containing unknown microbes can be contacted with the sensor

chip whereby one or more receptors of the bacteria react with

various different ligands tethered to the various sections of

the chip. Then, the fluorescence of the chip can be measured

with the probe 16 for the purpose of detecting which of the

sections of the sensor chip have analytes bonded thereto. As

examples, Myobacterium siderophores can be used to capture

mycobacteria such as mycobacterium tuberculosis. Helicobacter

pylori can be captured using tethered N-acetylneuroaminyl-alpha-

2,3-galactose. The peptide:

                    GADRSYLSFIHLYPELAGAC

can be tethered, by means of the terminal systeine group to

suppress capture Staphylococcus aureus toxic-shock toxin-1.

        As indicated above, some of the analytes of interest

can be identified by determining the presence of a single

captured microorganism, protein or peptide. In other cases,

however, a series of two or more captured analytes of interest

is indicative of the identity of a particular analyte. As an

example, consider a sensor chip having an area of three sections

along the horizontal axis and three sections along the vertical

axis as illustrated below:

-34-

A1	A2	A3
B1		
C1		

As an example, the sections identified can be provided with the following ligands tethered to each specific section as set forth in the following table:

Section Location	10x10 Array Ligand
A1	asialo G_{m1}
A2	hemin
A3	pyochelin
B1	GalNAcβGal
B2	alcaligin
B3	fibronectin peptide
C1	diferric transferrin
C2	staphyloferrin
C3	ferrioxamine B

It has been found that Pseudomonas aeruginosa can be identified as the microorganism when analytes are detected in sections A1, A2, A3, B1, C1 and C3. Similarly, Klebsiella Pneumoniae is detected when sections A2, B1, C1 and C3 have analytes captured thereon, and Serratia marescens is identified when sections A2 and C3 have analyte captured thereon. Similarly, Staphylococcus aureus can be identified when sections A2, B1, B3, C2 and C3 contain analyte captured thereon.

It will be understood that various changes and modifications can be made in the determination, procedure, formulation and use without departing from the spirit of the invention, especially as defined in the following claims.

What Is Claimed:

1.   A method for the taxonomic identification of
microorganisms comprising:

    (a)   contacting a microorganism-containing sample with

           a sensor chip, said sensor chip having a

           patterned array on the surface thereof containing

           a plurality of sections, with each section having

           bonded thereto a ligand capable of binding

           thereto a microorganism;

    (b)   directing electromagnetic radiation onto the

           surface of the sensor chip to ascertain the

           presence of a microorgansim bound to te ligand on

           each section; and

    (c)   taxonomically identifying the microorganism

           contained in the sample as a function of the

           combination of different ligands having a

           microorganism bound thereto.

2.   A method as defined in cliam 1 wherein each
ligand is tethered to the surface of the chip by means of a

coupling agent.

3.   A method as defined in claim 1 wherein the

coupling agent is an organosilane compound.

4.   A method as defined in claim 1 wherein the sensor

chip is formed with a glass surface.

5.   A method as defined in claim 1 wherein the chip

has a surface formed of a non-reactive plastic.

6.   A method as defined in claim 1 wherein the

ligands are selected from the group consisting of heme

compounds, siderophores, oligosaccharides and peptides.

7.   A method as defined in claim 1 wherein the

elecromagnetic radiation has a wavelength less than about 400 nm

to detect NAD[P]H as an indication of the presence of respiring

cells.

8.   A method as defined in claim 1 wherein the

electromagnetic radiation has an excitation wavelength within

the range of about 265 to 295 nm to detect the presence of

trypotophan as an indication of the presence of spores,

-38-

nonviable cells, exotoxins and peptides.

9.   A method as defined in claim 1 wherein the

electromagnetic radiation has an excitation wavelength within

the range of about 280 to about 310 nm to detect calcium

dipicolinate as an indication of the presence of spores.

10.   A method for the identification of

microorganisms, proteins and peptides comprising:

(a)   contacting an analyte-containing sample with a

sensor chip having a patterned array on the

surface thereof containing a plurality of

sections, with each section having bonded thereto

a ligand capable of binding a microorganism,

protein or peptide;

(b)   directing the electromagnetic radiation onto the

surface of the sensor chip to ascertain the

presence of a microorganism, protein or peptide

bound to the ligand of each section; and

(c)   identifying the microorgansim, protein or peptide

contained in the sample as a function of the

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combination of different ligands having a

microorgansim, protein or peptide bound thereto.

11.   A method as defined in claim 10 wherein the

analyte contains bacteria, viruses, protozoans, algae and fungi.

12.   A method as defined in claim 10 wherein each

ligand is tethered to the surface of the chip by means of a

coupling agent.

13.   A method as defined in claim 10 wherein the

coupling agent is an organosilane compound.

14.   A method as defined in claim 10 wherein the

ligands are selected from the group consisting of heme

compounds, siderophores, oligosaccarides and peptides.

15.   A sensor chip for use in the identification of

microorganisms, proteins and peptides comprising:

(a)   a substrate having a biocompatible, nonreactive

surface having a patterened array of sections

thereon; and

-40-

(b)   a plurality of ligands capable of capturing

different microorganisms, proteins and peptides

tethered to the surface of the substrate, with

different ligands being present on sections of

the substrate.

16.   A chip as defined in claim 15 wherein each ligand

is tethered to the surface of the chip by means of a coupling

agent.

17.   A chip as defined in claim 16 where the coupling

agent is an organosilane.

18.   A chip as defined in claim 15 wherein the surface

of the chip is a glass surface.

19.   A chip as defined in claim 15 wherein the ligands

are each selected from the group consisting of heme compounds,

siderophores, oligosaccarides, and peptides.

20.   Apparatus for the identification of

microorgansims, proteins and peptides using a sensor chip having

a patterned array of sections thereon and a plurality of ligands

tethered to the surface of the chip, with sections having

different ligands tethered thereto, comprising:

      (a)   means for directing electromagnetic radiation
            toward at least one of the sections of the chip
            to determine if that one section has a
            microorganism, protein and/or peptide captured
            thereto;

      (b)   at least one detector for electromagnetic
            radiation capable of converting the radiation
            into an electrical signal; and

      (c)   means for receiving electrical signals
            corresponding to the emission of electromagnetic
            radiation from said one section and electrical
            signals corresponding to the electromagnetic
            radiation reflected by said one section as a
            measure of the amount of microorgansim, protein
            and/or peptide captured on said one section.

     21.   Apparatus as defined in claim 20 wherein the
means for directing electromagnetic radiation is adapted to
direct electromagnetic radiation of at least two discrete bands
of wavelengths.

-42-

22. Apparatus as defined in claim 20 wherein the means for directing electromagnetic radiation includes at least two excitation filters.

23. Apparatus as defined in claim 20 wherein the means for directing electromagnetic radiation simultaneously directs said radiation toward a plurality of sections simultaneously.

24. Apparatus as defined in claim 20 wherein the means for directing electromagnetic radiation directs said radiation sequentially toward each of the sections of the chip.

FIG.1

1	2	3	4	5	6	7	8	9	10
11									
21									
31									
41									
51									
61									70

8

FIG.2

excitation light——▶
◀—— fluorescence

7   7

16

16

40

9

8

8

fluorescence pattern   detection system   chip
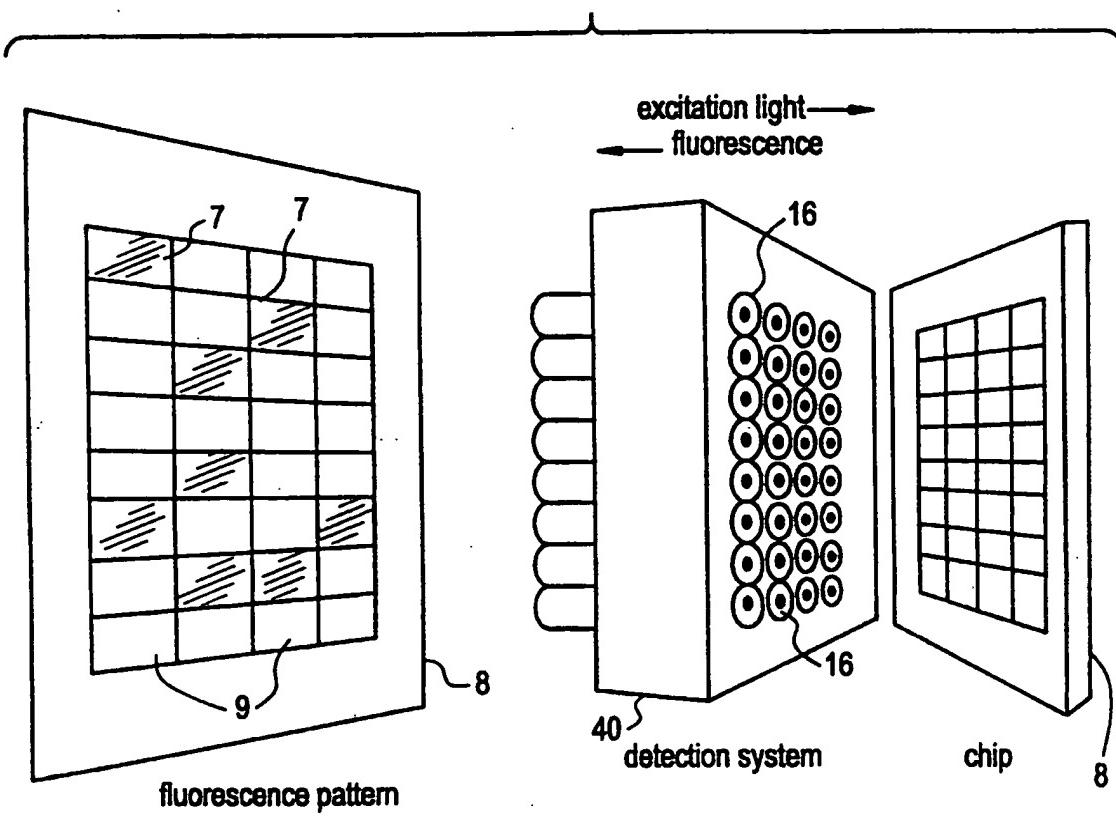
FIG.3
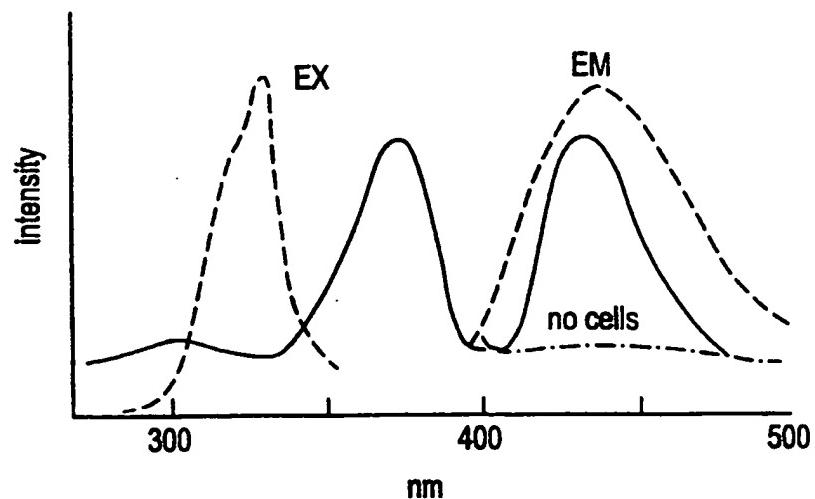
————— meat with E. coli and no fat

—·—·—·— meat with no fat and no E. coli

— — — — — meat with fat and no E. coli

FIG.4
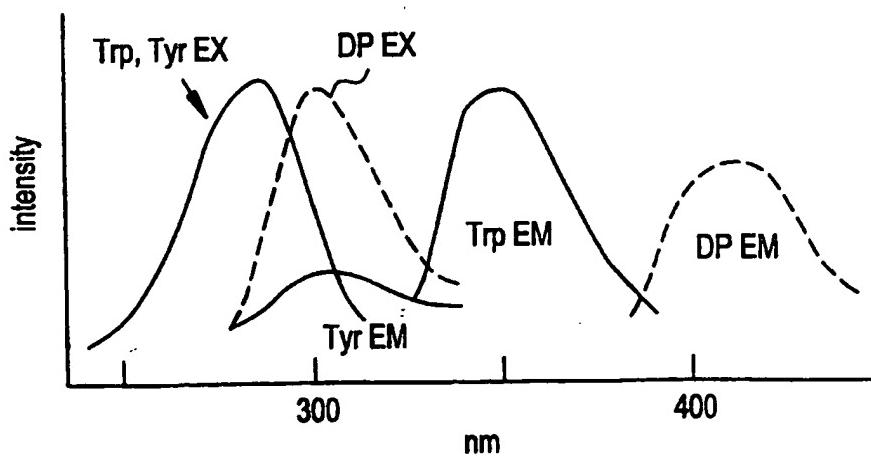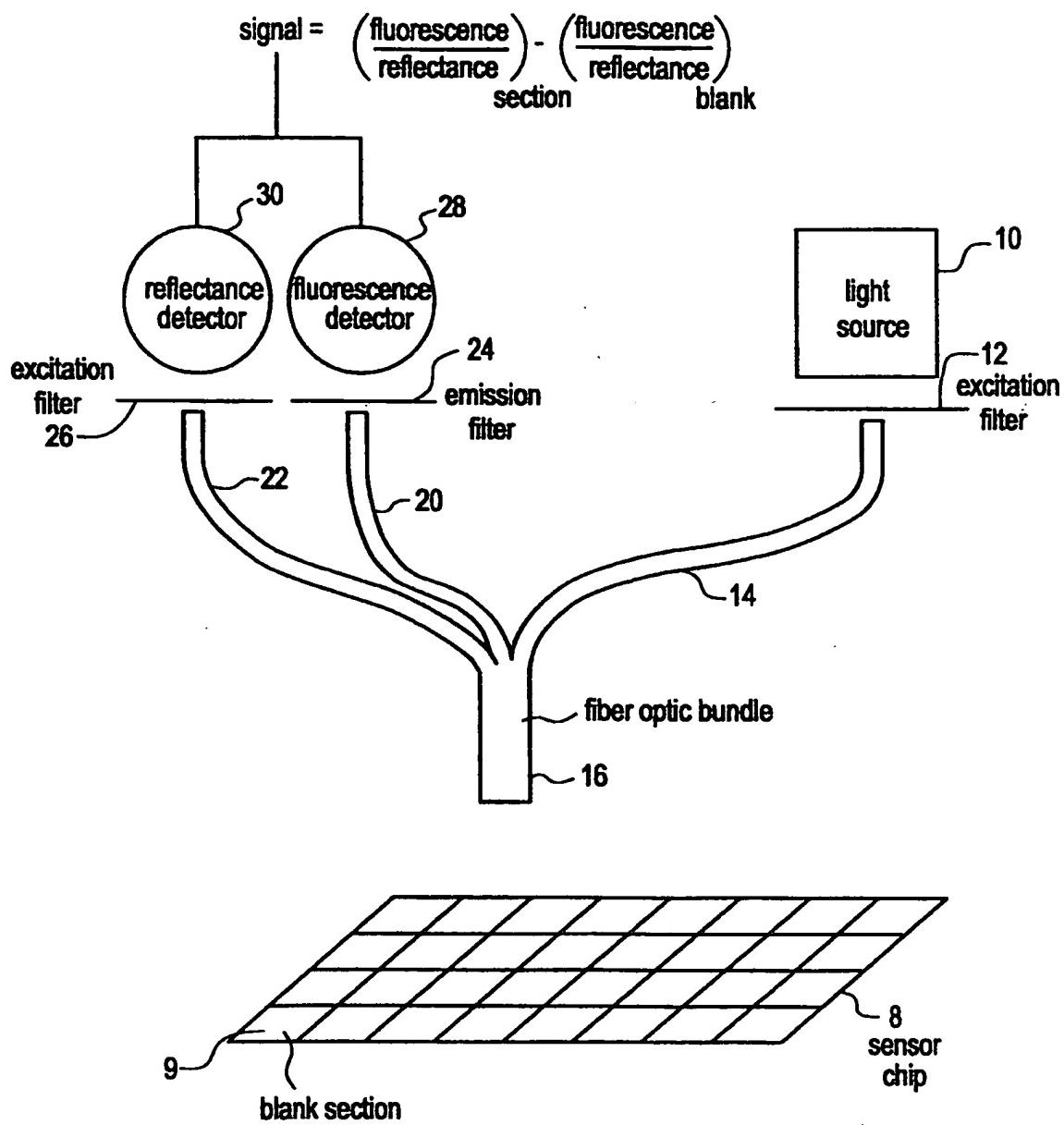
FIG.5

$$\text{signal} = \left(\frac{\text{fluorescence}}{\text{reflectance}}\right)_{\text{section}} - \left(\frac{\text{fluorescence}}{\text{reflectance}}\right)_{\text{blank}}$$

reflectance detector ~30

fluorescence detector ~28

light source ~10

excitation filter 26

emission filter 24

excitation filter ~12

~22

~20

~14

fiber optic bundle

~16

8 sensor chip

9

blank section

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/543   G01N33/569   G01N33/68   G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   G01N   C12Q   C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 276 968 A (YELLOWSTONE DIAGNOSTICS CORP) 3 August 1988 see the whole document	1-24
A	WO 94 24561 A (NILSSON & MANDENIUS) 27 October 1994 see abstract	1,10,15, 20
A	US 5 474 910 A (ALFANO ROBERT R) 12 December 1995 see the whole document	1,10,15, 20
A	US 4 631 413 A (JENSEN SVEND A K ET AL) 23 December 1986 see the whole document	1,10,15, 20

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 July 1998	04/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C

INTERNATIONAL SEARCH REPORT

	Inte. Jonal Application No
	PCT/US 98/08458

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 97 46867 A (B E SAFE INC ;POWERS LINDA (US)) 11 December 1997 cited in the application ---	1,10,15, 20
P,A	WO 97 27316 A (UNIV CALIFORNIA ;CHARYCH DEBORAH (US); NAGY JOHN (US)) 31 July 1997 see the whole document -----	1,10,15, 20

INTERNATIONAL SEARCH REPORT

Information on patent family members

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